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(54) **A process for the preparation of substituted tetrahydrofolic derivatives in the 6(R,S)(-) forms and of their active 6(S)(-) N5 diastereoisomers in form of alkali and earth alkali metal salts.**

(57) For the preparation of the [6(R,S)(-)]N<sup>5</sup> methyl-tetrahydrofolic acid and [6(R,S)(-)]N<sup>5</sup> formyl-tetrahydrofolic acids folic acid is hydrogenated with a strong excess of sodium borohydride at temperatures and for times controlled and made different between the admixing steps of the reactants and the true reduction reaction, whereafter the reaction product is treated with formic aldehyde and possibly subjected to a further reduction with NaBH<sub>4</sub>, if the methyl derivative is desired.

By having recourse to the salification with the chloride of an earth alkali metal the therapeutically active 6(S)(-) diastereoisomer is precipitated.

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The present invention relates to a novel industrial process for obtaining with optimum yields [6-(R,S)-]N<sup>5</sup> methyltetrahydrofolic acid or [6-(R,S)-]N<sup>5</sup> formyltetrahydrofolic acid isolated as Na, K, Li, Ca, Ba and Mg salts.

The invention further relates to a method for the separation of the two R and S forms aiming to obtain the active isomer, [6(S)-]N<sup>5</sup> methyltetrahydrofolic acid, but this method can be applied as well with good results to other tetrahydrofolic derivatives, differently substituted in the 5 position such as the 5-formyltetrahydrofolic acid and isolated as their salts of divalent inorganic ions.

The [6-(R,S)-]N<sup>5</sup> methyltetrahydrofolic acid can be considered as the first conversion metabolite of the [6-(R,S)-]N<sup>5</sup> formyltetrahydrofolic acid and is its biologically active form.

The pharmacological kinetics of the two stereoisomers has anyhow revealed that they are different since the (6S) isomer is absorbed in the gastrointestinal tract, probably thanks to a transport carrier, whereas a low biological activity has been assessed for the (6R) isomer.

Moreover the calcium [6(S)-]N<sup>5</sup> methyltetrahydrofolate, by passing the haematoencephalic barrier, represents the only form of folate which is actively carried into the central nervous system (CNS); as a matter of fact it is concentrated in the cephalic rachidian liquor and in the synaptic regions.

Furthermore on the basis of several experimental studies it results that this metabolite is also essential for the synthesis of S-adenosylmethionine in the CNS, by giving its methyl group to the homocysteine to get methionine which is thereafter converted into sulfoadenosylmethionine through the specific enzyme. Such a metabolic relationship between methyltetrahydrofolic acid and S-adenosylmethionine suggests a relevant antidepressive action of the methyltetrahydrofolic acid, since the synthesis of catecholaminic, indolaminic and imidazole neuromediators is promoted, the methylation deficit of the phospholipids in the geriatric age being moreover antagonized; in this manner its potential activity at the hepatocellular level is explained, the methylation processes being made easier also in the presence of hepatopathies.

In view of the above considerations there is foreseen the use of the [6(S)-]N<sup>5</sup> methyltetrahydrofolic acid in patients suffering of mental deterioration, as regards the induction of the memory and of knowledge functions, the behaviour and affective modifications as well as all that relates to the side symptoms of the pathologies related to the cerebrovascular apparatus of aged persons and of the hepatic malfunctions.

The purpose of the present invention is thus that of providing an industrially advantageous process for the preparation of [6-(R,S)-]N<sup>5</sup> methyltetrahydrofolic acid and [6-(R,S)-]N<sup>5</sup> formyltetrahydrofolic acids, of their salts with alkali and earth alkali metals as well as for the separation of their pharmaceutically active isomers, namely [6-(S)-]N<sup>5</sup> methyltetrahydrofolic acid and [6(S)-]N<sup>5</sup> formyltetrahydrofolic acid.

The present invention provides for the preparation of the above mentioned acids the following steps:

a) hydrogenation of an aqueous solution of folic acid with NaBH<sub>4</sub>, used at the concentrations as detailedly indicated hereinafter with respect to the method, in alkaline aqueous solution: the reaction is carried out at a temperature of between 5°C. and 30°C. and for a time of between 5 and 60 minutes for the mixing phase, whereas the reduction step is carried out at a temperature of between 70°C. and 100°C. and for a time of 20 to 30 minutes.

b) reaction of the product from the reaction step (a) with formic aldehyde at a temperature less than 90°C. and then reduction with an alkaline aqueous solution of sodium borohydride at a temperature of the order of 60°C.

c) separation through precipitation of the mixture of inorganic salts by treatment with a strong acid and adjustment of the pH of the resulting solution at a value close to the neutrality, which represents a fundamental feature for obtaining the final product.

As regards the preparation of the salts of earth alkali metals the present invention comprises

d) adding to the solution of the step (c) an aqueous solution of a chloride of the desired earth alkali cation, the desired salt being then separated by crystallization at a pH of between 7 and 7.2

Lastly the separation of the desired active isomer is carried out according to the present invention by a process comprising the instantaneous addition to the solution obtained in the previous step (c), maintained at pH 7 and at a controlled temperature of between -5°C. and +10°C., and preferably of between -2°C. and +2°C., a 4M aqueous solution of CaCl<sub>2</sub>, the calcium salt of the desired isomer, namely calcium [6(S)-]N<sup>5</sup> methyl or formyl tetrahydrofolate, being then crystallized, this salt being pure and devoid of the R isomer; the precipitated product is in form of a mixture of monohydrate and pentahydrate crystalline forms.

More details about the process of the present invention can be obtained from the following exemplifying description of the single process steps.

1. Preparation of the [6(R,S)(-)]N<sup>5</sup> methyl-tetrahydrofolic acid.

A 500 litres enameled reactor (A) is used, having a cooling and heating jacket, means being provided for the stirring, for the bubbling of pure nitrogen, together with a thermometer and with means for the possible direct connection with a reactor (B), having a cooling jacket and a stirrer.

The reactor (A) is charged with 14 kg of NaBH<sub>4</sub> in 17.4 litres of a 20% aqueous solution of NaOH under stirring and keeping the temperature at a controlled level of +20°C.

Then 0.5 kg of disodium salt of ethylenediaminetetraacetic acid (EDTA) are added. In this reaction mixture pure nitrogen is bubbled with a continuous flow and the reaction is continued under these conditions. The internal temperature of the reaction mass is lowered to between 0°C. and +5°C.

60 litres of water are charged in the reactor (B) and under stirring there are added in portions 20 kg of folic acid of the F.U. type, the suspension being vigorously stirred and then the reaction mixture is cooled to +5°C. This solution (B) is poured in the reactor (A) in a time of between 5 and 60 minutes, preferably of between 20 and 30 minutes, taking care that the internal temperature of the reaction is maintained within the 5-30°C. interval, preferably between 20°C. and 25°C.

Upon the addition is completed the reaction mixture is heated up to 90-95°C. for a time of 20 minutes (step A).

The mixture is cooled to 15°C. and then in about 30 minutes and at a temperature not higher than 20°C. 15.4 litres of 37% HCl are added.

The final pH after the pouring is about 9. The temperature is brought back to less than 10°C.

Then in the reactor (A) 9 litres of formic aldehyde are slowly added, the temperature being always maintained to less than 30°C. The reaction is highly exothermic and the temperature in this step is to be very carefully controlled since it really determines the obtainable final yields. At a reactor temperature of 5°C. the solution present in the reactor (A) is added with a cooled solution of 7 kg of NaBH<sub>4</sub> suspended in 10 litres of 0.2N NaOH in water; the addition is to be effected in 20 minutes taking care that the temperature also in this phase is not higher than 20°C.

Upon the addition is completed the mixture is very slowly heated to 60°C. and this temperature is maintained for a 15 minutes time.

The resulting mixture is cooled to +5°C., 9.2 litres of 37% HCl are added and the temperature is maintained for 2 hours at a value below and not higher than 5°C. to let the inorganic salts consisting of borates to be crystallized.

The precipitate of inorganic salts is filtered and the resulting solution is added with about 1.6 litres of 37% HCl so that the pH is between 7.1 and 7.2 (solution X) (step B).

2. Precipitation of [6(R,S)(-)]N<sup>5</sup> methyl-tetrahydrofolates diastereoisomers as calcium salts.

The solution X as above obtained is added with 40 litres of a 2M CaCl<sub>2</sub> solution, care being taken of controlling that the final pH is 7 (this fact representing an essential feature for the prosecution of the reaction).

The temperature of this solution is lowered to +10°C. and the product is let to crystallize.

After filtration by centrifugation a recrystallization is carried out in 200 litres of boiling water.

2 kg of active carbon are added, if necessary, and the mixture is filtered. The solution is cooled to +10°C. and the final product is let to crystallize until it is completely precipitated.

The product is filtered and dried in a drier under vacuum avoiding light and contact with oxygen. The yield of the final product is 14 kg.

Like results are obtained in the preparation of the respective Mg and Ba folates, the corresponding chlorides MgCl<sub>2</sub> and BaCl<sub>2</sub> being used as 32 litres of a 2M solution.

The Na, K and Li salts are obtained by using the proper ion exchange resins from Ca, Mg and Ba salts.

3. Precipitation of the active isomer, [6(S)(-)]N<sup>5</sup> methyltetrahydrofolic acid as the calcium salt.

The solution X as above obtained in (1) and carefully adjusted to pH 7 and to a temperature of between +2°C. and -2°C. is added in one shot with a solution of 5 litres of CaCl<sub>2</sub> in water with a 4M concentration.

The crystallization of the product is very slow and takes place in a time of 4-5 days, the solution being maintained at a constant temperature of between +2°C. and -2°C.

Calcium [6(S)(-)]N<sup>5</sup> methyltetrahydrofolate is precipitated as the product in an amount of between 1,800 and 2,200 kg.

The product is found pure and devoid of R isomer.

It can be recrystallized from boiling water and has the following chemical and physical characteristics:

Raw formula: C<sub>2</sub>OH<sub>2</sub>3N<sub>7</sub>O<sub>6</sub>Ca M.W.: 497.55

Aspect: white brown crystalline powder

Moisture: 15.27%

Solubility: soluble in acids and bases; practically insoluble in water; insoluble in organic solvents.

Calcium content: 8.07%

HPLC purity: 99.0%

U.V. (20 mg/l in 1%  $\text{NH}_4\text{OAc}$ /pH 6.0) = 290 nm (= 28400) max min = 245 nm  $\text{Al/Al}$  = 3.7

Stereoisomeric purity: 99.0% (6S)(-)

4. Preparation of the [6(R,S)(-)] $\text{N}^5$  formyltetrahydrofolic acid

The process is carried out up to the step A as for the methyltetrahydrofolic acid.

Then the mixture is made acidic with HCl up to acid pH whereby tetrahydrofolic acid precipitates.

The latter is suspended up to neutral pH and the thus obtained solution is added with formic aldehyde. The mixture is stirred for 2 hours at 10°C., then a stoichiometrical amount of  $\text{CaCl}_2$  is added and the [6(R,S)(-)] $\text{N}^5$  formyltetrahydrofolic acid is crystallized from a solution oversaturated by NaCl.

5. Separation of mixtures of stereoisomers of [6-(R,S)(-)] $\text{N}^5$  formyltetrahydrofolic acid as the calcium salt of the active isomer, [6(S)(-)] $\text{N}^5$  formyltetrahydrofolic calcium salt.

600 g of a mixture of stereoisomers of [6-(R,S)(-)] $\text{N}^5$  formyltetrahydrofolic acid as the pentahydrate calcium salt are added to 1,500 litres of water under nitrogen stream, the mass being maintained under stirring and at a temperature of between 5 and 10°C.

370 g of EDTA disodium salt are added in portions under stirring at the same time as a 10M solution of sodium hydroxide in stoichiometrical amount with respect to the weighed amount of stereoisomers. The solution is maintained under stirring in these conditions for about one hour, by carefully controlling that the pH of the solution is 7. Then 33 g of anhydrous  $\text{CaCl}_2$  are added in portions. The pH is controlled at a value of between 6.7 and 7.2.

The solution is made oversaturated by adding NaCl and then is stored in a refrigerator at a temperature of between -5°C. and +10°C., preferably between +2°C. and -2°C.

It is maintained at rest for 4 days and the precipitate is collected in an amount of 120 g of [6(S)(-)] $\text{N}^5$  formyltetrahydrofolic stereoisomer. It is recrystallized from ethanol/water and there are obtained 100 g of pure product, having at the HPLC control the chemical and physical properties of the standard sample. The above procedure is repeated until a product having the desired isomeric purity is obtained.

By the same process the active stereoisomer [6(S)(-)] $\text{N}^5$  methyltetrahydrofolic having the same above mentioned chemical and

physical properties can be prepared from mixtures of calcium salts of [6(R,S)(-)] $\text{N}^5$  methyltetrahydrofolic acid.

## 5 Claims

1. A process for the preparation of [6(R,S)(-)] $\text{N}^5$  methyltetrahydrofolic acid and [6(R,S)(-)] $\text{N}^5$  formyltetrahydrofolic acids, of their salts with alkali and earth alkali metals as well as for the separation of their pharmaceutically active isomers, namely [6(S)(-)] $\text{N}^5$  methyltetrahydrofolic acid and [6(S)(-)] $\text{N}^5$  formyltetrahydrofolic acid, characterized by the following steps:

a) hydrogenation of an aqueous solution of folic acid with  $\text{NaBH}_4$ , used at an excess concentration, preferably as a very high excess, with respect to the necessary stoichiometrical amount, in alkaline aqueous solution, the reaction being carried out at a temperature of between 5°C. and 30°C. and for a time of between 5 and 60 minutes for the mixing phase, whereas the reduction step is carried out at a temperature of between 70°C. and 100°C. and for a time of 20 to 30 minutes;

b) reaction of the product from the reaction step (a) with formic aldehyde at a temperature less than 90°C. and

b1) reduction with an alkaline aqueous solution of sodium borohydride at a temperature of the order of 60°C. when the obtention of [6(R,S)(-)] $\text{N}^5$  methyltetrahydrofolic acid is desired, or

b2) the reaction with formic aldehyde being preceded by an acidification adapted to cause the tetrahydrofolic acid to precipitate when the obtention of [6(S)(-)] $\text{N}^5$  formyltetrahydrofolic acid is desired, and

c) separation through precipitation of the mixture of inorganic salts by treatment with a strong acid and adjustment of the pH of the resulting solution at a value close to the neutrality.

2. A process according to claim 1, characterized in that the solution resulting from the step (c) is added with an aqueous solution of a halide of the desired alkaline or earth alkaline cation, the desired salt being then separated by crystallization at a pH of between 7 and 7.2.

3. A process according to claim 2, characterized in that a 2M aqueous solution of calcium chloride is used.

4. A process according to claim 1, characterized in that in order to obtain the optically active [6-(S)(-)] diastereoisomer of the said acids the solution obtained from the step (c), maintained at pH 7 and at a controlled temperature of between -5°C. and +10°C., is instantaneously added with a 4M aqueous solution of the chloride of the earth alkaline cation, the salt of the earth alkali metal of the desired isomer being then let to crystallize.
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5. A process according to claim 4, characterized in that said solution obtained from the step (c) is maintained at a controlled temperature of between -2°C. and +2°C. and the said salt of earth alkali metal is calcium, barium or magnesium chloride.
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6. A process according to claim 1, characterized in that said hydrogenation of the folic acid is carried out by adding an aqueous solution of folic acid, maintained at a temperature of 5°C., to a 20% NaOH water solution in which the strong excess of NaBH<sub>4</sub> is dissolved under stirring and is maintained at 20°C., said solution being added with disodium salt of ethylenediaminetetraacetic acid(EDTA) and brought to a temperature of between 0°C. and 5°C.
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7. A process according to claim 1, characterized in that in said mixing phase the temperature is of between 20°C. and 25°C.
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(57) For the preparation of the [6(R,S)(-)]N<sup>5</sup> methyltetrahydrofolic acid and [6(R,S)(-)]N<sup>5</sup> formyltetrahydrofolic acids folic acid is hydrogenated with a strong excess of sodium borohydride at temperatures and for times controlled and made different between the admixing steps of the reactants and the true reduction reaction, whereafter the reaction product is treated with formic aldehyde and possibly subjected to a further reduction with NaBH<sub>4</sub>, if the methyl derivative is desired.

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## EUROPEAN SEARCH REPORT

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### DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. CL.5)
A	EP-A-0 189 990 (DANA-FARBER CANCER INSTITUTE) *Insgesamt*	1-7	C07D475/04
A	GB-A-1 572 138 (BIORESEARCH S.A.S. DEL DR. LIVIO CAMOZZI) *Insgesamt*	1-7	
A	CH-A-635 344 (BIORESEARCH) *Insgesamt*	1-7	
P,A	US-A-5 124 452 (FEDERICO GENNARI) *Insgesamt*	1-7	
			TECHNICAL FIELDS SEARCHED (Int. CL.5)
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The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 04 MARCH 1993	Examiner LUYTEN H.W.
CATEGORY OF CITED DOCUMENTS			
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